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# EDG8 RECEPTOR, ITS PREPARATION AND USE

This application claims priority to European Patent Application Nos. 108858.2, filed April 26, 2000, and 116589.3, filed August 1, 2000, the disclosures of which are hereby incorporated by reference in their entireties.

## FIELD OF THE INVENTION

The present invention relates to newly identified human EDG8 receptors, polynucleotides encoding this receptor, polypeptides encoded by such polynucleotides, the preparation and the use of such polynucleotides and polypeptides.

## BACKGROUND OF THE INVENTION

In an effort to identify new G-protein coupled receptors of the EDG (endothelial differentiation gene)-family a novel member of the EDG-family of G-protein coupled receptors, Human EDG8, was identified. The full-length clone was isolated and studies on chromosomal mapping, tissue expression and identification as a functional cellular receptor for sphingosine 1-phosphate were performed. Taken together, the data provide compelling evidence that EDG8 is the fifth receptor for sphingosine 1-phosphate, exclusively expressed in peripheral tissues, its presence in endothelial cells being responsible for the broad tissue distribution.

The lysolipid phosphate mediators lysophosphatidic acid (LPA) and sphingosin 1-phosphate (S1P) have attracted increasing attention as modulators of a variety of important biological functions (Moolenaar et al., Current Opinion in Cell Biol 9:168, 1997; Morris, Trends Pharmacol Sci 20:393, 1999; Lynch and Im, Trends in

Pharmacol Sci 20:473, 1999) and their list of biological activities is continuously growing.

Among the biological responses to LPA is platelet aggregation (Jalink et al., Biochem Biophys Acta 1198:185, 1994; Siess et al., PNAS USA 96:6931, 1999; Gueguen et al., Biochemistry 38: 8440, 1999), smooth muscle contraction (Tokumura et al., Arch Int Pharmacodyn Ther 245:74, 1980), in vivo vasoactive effects (Tokumura et al., Res Comm Mol Pathol Pharmacol 90:96, 1995), chemotaxis (Jalink et al., PNAS USA 90:1857, 1993), expression of adhesion molecules (Lee et al., J Biol Chem 273:22105, 1998; Rizza et al., Laboratory Investigation 79:1227, 1999), increased tight junction permeability of endothelial cells (Schulze et al., J Neurochem 68:991, 1997), induction of stress fibers (Gohla et al., J Biol Chem 274:17901, 1998) and many others (for review see Moolenaar et al., Current Opinion in Cell Biol 9:168, 1997). The biochemical signalling events that mediate the cellular effects of LPA include stimulation of phospholipases, mobilization of intracellular $Ca^{2+}$, inhibition of adenylyl cyclase, activation of phosphatidylinositol 3-kinase, activation of the Ras-Raf-MAP kinase cascade and stimulation of Rho-GTPases (Moolenaar et al., Current Opinion in Cell Biol 9:168, 1997).

S1P, in particular, is implicated in cell proliferation, modulation of cell motility (reviewed in Hla et al., Biochem Pharm 58:201, 1999) induction/suppression of apoptosis (Hisano et al., Blood 93:4293, 1999; Xia et al., J Biol Chem 274:34499, 1999), angiogenesis (Lee et al., Cell 99:301, 1999), tumor invasiveness (Sadahira et al., PNAS USA 89:9686, 1992), platelet activation (Gueguen et al., Biochemistry 38: 8440,1999) and neurite retraction (Postma et al., EMBO J 15:2388, 1996). Cellular signalling by S1P involves activation of PLCß and subsequent intracellular $Ca^{2+}$ release (van Koppen et al., J Biol Chem 271:2082, 1996; Meyer zu Heringdorf et al., Naunyn-Schmiedeberg's Arch Pharmacol 354:397, 1997; Yatomi et al., J Biol Chem 272:5291, 1997a; Noh et al., J Cell Physiol 176:412, 1998; Ancellin and Hla, J Biol Chem 274:18997, 1999), activation of MAP-kinases (Wu et al., J Biol Chem 270:11484, 1995; Lee et al., J Biol Chem 271:11272, 1996; An et al., J Biol Chem

275:288, 2000), activation of inward rectifying $K^+$-channels (van Koppen et al., J Biol Chem 271:2082, 1996; Bünemann et al., EMBO J 15:5527, 1996) and inhibition and/or activation of adenylyl cyclase (Lee et al., J Biol Chem 271:11272, 1996).

Both, LPA and S1P are recognized to signal cells through a set of G-protein coupled receptors (GPCRs) known as EDG (endothelial differentiation gene)-receptors. The EDG-family of GPCRs currently comprises seven human members (EDG1-7) that fall into two major groups depending on their preference for the activating lipid-ligand: EDG1, 3, 5 and 6 preferentially interact with S1P (Yatomi et al., J Biochem (Tokyo) 12:969, 1997; Lee et al., Science 279:1552, 1998; Lee et al., J Biol Chem 273:22105, 1998; Ancellin and Hla, J Biol Chem 274:18997, 1999; Yamazaki et al., Biochem Biophys Res Commun 268:583, 2000; Van Brocklyn et al., Blood 95:2624, 2000), EDG2, 4 and 7 preferentially interact with LPA (An et al., J Biol Chem 273:7906, 1998; Im et al., Mol Pharmacol 57:753, 2000 ).

The assignment of specific biological functions to certain receptor subtypes is hampered by the fact that EDG receptors are expressed in an overlapping fashion (Rizza et al., Laboratory Investigation 79:1227, 1999; Lee et al., Cell 99:301, 1999), they activate multiple and in part redundant signal transduction pathways (Lee et al., J Biol Chem 271:11272, 1996; Ancellin and Hla, J Biol Chem 274:18997, 1999; Kon et al., J Biol Chem 274:23940, 1999; An et al., J Biol Chem 275:288, 2000), the selectivity for their activating ligands is not absolute (Lee et al., J Biol Chem 273:22105, 1998), and medicinal chemistry is only poorly developed in that specific antagonists for dissecting the pharmacology of the individual subtypes are not available yet.

An important step to shed more light on the biological role of the individual receptor subtypes would be to identify the complete set of receptors that respond to the phospholipid mediators S1P and LPA.

SUMMARY OF THE INVENTION

The present invention relates to newly identified human EDG8 receptors, polynucleotides encoding this receptor, polypeptides encoded by such polynucleotides and the preparation and the use thereof.

The present invention relates to an isolated polynucleotide comprising a nucleotide sequence that has at least 90 % identity, preferably 95 % or more, most preferably 98 % identity to a nucleotide sequence encoding the polypeptide of SEQ ID NO:2 or the corresponding fragment thereof; or a nucleotide sequence complementary to said nucleotide sequence.

The present invention also relates to an isolated polynucleotide comprising a nucleotide sequence that has at least about 90 % identity, preferably about 95 % or more, most preferably about 98 % identity to a nucleotide sequence encoding the polypeptide of SEQ ID NO:2 or the corresponding fragment thereof; or a nucleotide sequence complementary to said nucleotide sequence.

Preferably, the polynucleotide is DNA or RNA. The nucleotide sequence of the polynucleotide is at least 90 % or about 90% identical to that contained in SEQ ID NO:1; preferably 95 % or about 95% or more, most preferred 98 % or about 98% or more identical to SEQ ID NO:1. In another embodiment, the polynucleotide has the nucleotide sequence SEQ ID NO:1. In another embodiment, the polynucleotide encodes the polypeptide of SEQ ID NO:2 or a fragment thereof. In a special embodiment, the polynucleotide is an allele of SEQ ID NO:1. Preferably, the polynucleotide has the same essential properties and/or biological functionality as human EDG8.

One characteristic of "functionality" or "biological functionality" is that the polynucleotide encodes for a S1P receptor; it responds to S1P and optionally also to related phospholipids like DMS 1P or LPA. By "functionality" is meant the molecule is a functional receptor for S1P, LPA, dHS1P and related lysophospholipid mediators. Such activity may be assayed using well known techniques in the art. One such assay employs assessment of ability of $Ca^{2+}$ mobilization in response to

S1P mediated by the receptor, e.g., EDG8 or a functional fragment thereof, in CHO cell as set forth in the description of Figure 2.

Another aspect of the invention relates to an expression system for the expression of EDG8. The EDG8 DNA or RNA molecule comprising an expression system wherein said expression system is capable of producing a polypeptide or a fragment thereof having at least 90 % or about 90% identity, preferably 95 % or about 95% or more, most preferred 98 % or about 98% or more identity with a nucleotide sequence encoding the polypeptide of SEQ ID NO. 2 or said fragment when said expression system is present in a compatible host cell. Preferably, the expression system is a vector.

The invention relates to a host cell comprising the expression system.

In another aspect, the invention relates to a process for producing a human EDG8 polypeptide or a fragment thereof wherein a host cell comprising the expression system is cultured under conditions sufficient for the production of said polypeptide or fragment thereof. Preferably, the said polypeptide or fragment thereof is expressed at the surface of said cell.

The invention relates also to cells produced by this process.

The process preferably further includes recovering the polypeptide or fragment thereof from the culture.

In another aspect, the invention relates to a process for producing a cell which produces an EDG8 polypeptide or a fragment thereof comprising transforming or transfecting a host cell with the expression system such that the host cell, under appropriate culture conditions, produces a human EDG8 polypeptide or a fragment thereof.

Thus, in one embodiment, the invention relates to an isolated polynucleotide comprising a polynucleotide selected from the group consisting of:

(a) a polynucleotide encoding the polypeptide consisting of the amino acid sequence of SEQ ID NO:2;

(b) a polynucleotide consisting of SEQ ID NO:1;

(c) a polynucleotide having at least about 90% sequence identity to the polynucleotide of (a) or (b).

In another embodiment, the invention relates to a fragment of the polynucleotide of SEQ ID NO:1. In yet another embodiment, the invention relates to a polynucleotide which is a complement of the above described polynucleotide.

Other embodiments relate to an expression vector comprising the isolated polynucleotide and a host cell comprising such expression vector. A further embodiment is a method of producing a polypeptide comprising SEQ ID NO:2 by culturing such host cell under conditions sufficient for the production of the polypeptide and recovering it from the culture. Another embodiment of the invention relates to a process for producing cells capable of expressing the above polypeptide comprising genetically transfecting or transforming cells with the above vector.

Another embodiment relates to an antibody that selectively binds a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or a fragment of SEQ ID NO:2.

A further embodiment relates to a process for diagnosing a disease or a susceptibility to a disease related to expression or activity of human EDG8 polypeptide comprising:

determining the presence or absence of mutation in the nucleotide sequence encoding human EDG8 polypeptide in the genome of the subject; and/or

analyzing for the presence or amount of the human EDG8 polypeptide expression in a sample derived from the subject.

Another embodiment relates to a method for identifying compounds which bind to human EDG8 polypeptide comprising:

a) contacting a cell containing the above described polynucleotides of the invention with a candidate compound; and

b) assessing the ability of said candidate compound to bind to the cells. This method further includes determining whether the candidate compound effects a signal generated by activation of the human EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of the signal is identified as an agonist. In another embodiment, this method further includes determining whether the candidate compound effects a signal generated by activation of the human EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of the signal is identified as an antagonist.

Thus, the present invention relates to agonists and antagonists identified by the above described methods.

In yet another embodiment, the invention relates to a method of preparing a pharmaceutical composition comprising:

a) identifying a compound which is an agonist or an antagonist of human EDG8,

b) preparing the compound, and

c) optionally mixing the compound with suitable additives and to pharmaceutical composition prepared by such method.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1A: The nucleotide and deduced amino acid sequence of human EDG8. The deduced amino acid sequence is shown below the nucleotide sequence with the nucleotide positions indicated on the left.

Fig. 1B: Phylogenetic tree of the EDG-family of receptors. The phylogenetic tree depicted was derived by the neighbor joining method performed with the GCG program Wisconsin package version 10.1-Unix (Genetic Computer Group (GCG), Madison, Wisconsin.

Fig.1C: Alignment of the amino acid sequence of human EDG8 with the other EDG-family members. The amino acid sequence of human EDG8 (accession number AC011461) is compared with the EDG1-7 polypeptides (EDG1: accession number M 31210, EDG2: accession number U 80811, EDG3: accession number X 83864, EDG4: accession number AF 011466, EDG5: accession number AF 034780, EDG6: AJ 000479, EDG7: accession number AF 127138). The approximate boundaries of the seven putative transmembrane domains are boxed. Gaps are introduced to optimize the alignment.

Fig.2A-F: Mobilization of intracellular $Ca^{2+}$ by S1P (10, 100 and 1000 nM) mediated by the EDG1, 3, 5, 6 and 8 receptor in CHO cells, cotransfected with empty vector DNA as a control or the indicated G-protein α subunits.

A: S1P-induced $Ca^{2+}$-response in CHO cells transfected with vector DNA alone or the G protein α subunits Gq, G16 and Gqi5. B-F: S1P-induced $Ca^{2+}$-response in CHO cells transfected with the indicated EDG-receptor subtypes. Agonist-mediated changes of intracellular $Ca^{2+}$ were measured with the FLIPR using the $Ca^{2+}$-sensitive dye FLUO4 as described in Experimental procedures. Fluorescence of transfected cells loaded with FLUO4 was recorded before and after addition of S1P, applied in the indicated concentrations. Data are expressed as means of quadruplicate determinations in a single experiment. An additional experiment gave similar results.

Fig.3: Effects of S1P, LPA and related lysophospholipid mediators on EDG8-mediated increase in intracellular $Ca^{2+}$. CHO-cells were cotransfected with EDG8 and the G protein α subunits Gqi5 (upper panel) and G16 (lower panel) and rises in $[Ca^{2+}]_i$ were recorded with the FLIPR as described in Experimental procedures. The different lipids were applied in concentrations of 10, 100 and 1000 nM,

respectively. Data are means of quadruplicate determinations of a representative experiment. Two additional experiments gave similar results.

Fig.4: Northern blot analysis of EDG8 in human tissues. Poly(A)+ RNA (1µg) from various human tissues (human multiple tissue Northern blots, CLONTECH) was hybridized with probes specific to human EDG8 (upper panel) and ß-actin (lower panel) on a nylon membrane. The origin of each RNA is indicated at the top, the molecular mass of standard markers in kilobases (kb) is shown on the left.

Fig.5A: Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of EDG8 in different human endothelial cell lines (HUVECs: human umbilical vein endothelial cells; HCAEC: human coronary artery endothelial cells; HMVEC-L: human microvascular endothelial cells from lung; HPAEC: human pulmonary artery endothelial cells). EDG8-specific transcripts were detected in all endothelial cell lines. Agarose gel electrophoresis of the PCR products after 35 cycles of amplification with the GC-melt kit (as described in Experimental Procedures) is shown. Amplification with EDG8-specific primers yields a 522 bp EDG8-fragment as indicated by the arrow. The EDG8 plasmid served as a template for the positive control, $H_2O$ was used instead of plasmid DNA as a negative control.

Fig.5B: PCR analysis of EDG8 primers for specificity of amplification of EDG8 sequences. Primers, specific for the EDG8 sequence, were checked for potential amplification of the related EDG1-7 sequences, using the respective plasmids as templates. Agarose gel electrophoresis of the PCR products after 35 cycles of amplification with the GC-melt kit (as described in Experimental Procedures) is shown. The EDG8 specific 522 bp band occurred only when EDG8 was used as a template. $H_2O$ was used instead of plasmid DNA as a negative control.

Fig.6: Experiments were performed according to example 3. Instead of lipids, a lipid library was used.

Fig.6A+B: Library plattes with rat EDG8 (r EDG8) and qi5.

Fig.6A: qi5 background.

Fig.6B: Measurement with rEDG8.

Fig.6C: Fluorescence change counts.

Fig.7: Experiments were performed according to example 3. Instead of Lipids, a lipid library was used.

Fig.7A+B: Library plates with human EDG8 (hEDG8) and qi5.

Fig.7A: q15 background.

Fig.7B: Measurement with hEDG8

Fig.7C: Fluourescence change counts.

Fig. 8: Antagonism of S1P activation of rat and human EDG8. Transiently transfected CHO cells expressing rat EDG8 and $G\alpha_{qi5}$ **(A)** and HEK 293 cells expressing human EDG8 and $G\alpha_{qi5}$ **(B)** were incubated with test compounds, namely, 0.1µM Leukotriene B4, 1µM 2-DHLA-PAF (1-O-Hexadecyl-2-O-dihomo-γ-linolenoyl-sn-glycero-3-phophorylcholine), 1µM $C_2$ Dihydroceramide, 0.1µM 15(S) HEDE (15(S)-Hydroxyeicosa-11Z,13E-dienoic acid), 1µM PAF C16 (1-O-Hexadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine), 1µM 16,16 Dimethyl $PGE_2$ (16,16-Dimethyl-Prostaglandin $E_2$) 12, 0.1µM (R)-HETE (12(R)-Hydroxyeicosa-5Z,8Z,10E,14Z-tetraenioc acid), 1µM 8-epi-$PGF_{2\alpha}$ (8epi-Prostaglandin $F_{2\alpha}$ ) 0.1µM Leukotoxin A ((±) 9,10-EODE) or with solvent buffer for 3 min and then challenged with 1µM S1P (sphingosine 1-phosphate). Peak fluorescence counts of cells preincubated with solvent buffer and then stimulated with 1µM S1P were set 100 %. Fluorescence change counts were recorded with the FLIPR as described in detail in Experimetal procedures. Data are means ± SE of 2-3 independent experiments.

Fig. 9: Inhibition of S1P mediated intracellular calcium release by suramin and NF023 (8,8`-(carbonylbis(imino-3,1-phenylene))bis-(1,3,5-naphatlenetrisulfonic acid)) in cells transiently cotransfected with with human EDG8 and $G\alpha_{qi5}$ **(A)** and rat EDG8 and $G\alpha_{qi5}$ **(B)**. Transfected cells were first treated with the indicated concentrations of the inhibitor or solvent buffer for 3 minutes (NF023 and suramin did not show any effect on $[Ca^{2+}]_i$ mobilization during the preincubation period). Cells were then stimulated with 1µM S1P and in $[Ca^{2+}]_i$ measured with the FLIPR as described in the method section. Peak fluorescence counts were normalized and background responses of $G\alpha_{qi5}$ -transfected cells were subtracted. S1P-mediated calcium release in the absence of inhibitor was set 100%. Data are means ± SE of 4-7 independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

The abbreviations used are:

S1P, sphingosine 1-phosphate; LPA, lysophosphatidic acid; dHS1P, dihydro sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; LPC, lysophosphatidylcholine; GPCR, G-protein-coupled receptor; G-protein, guanine nucleotide-binding protein; $[Ca^{2+}]_i$, intracellular Calcium concentration, RT-PCR, reverse transcription polymerase chain reaction; bp, base pair; ORF, open reading frame; EST, expressed sequence tag; FAF-BSA, fatty acid free bovine serum albumine; HUVECs, Human umbilical vein endothelial cells; HCAECs, human coronary artery endothelial cells; HMVEC-L, human microvascular endothelial cells from lung; HPAEC, human pulmonary artery endothelial cells.

In particular, the invention relates to an EDG8 polypeptide or a fragment thereof comprising an amino acid sequence which has at least about 90 %, preferably at least about 95 %, most preferred about 98 % or more identity to the amino acid sequence SEQ ID NO. 2 or to a part of SEQ ID NO. 2. In particular the invention relates to an EDG8 polypeptide or a fragment thereof having amino acid sequence SEQ ID NO. 2 or a part thereof. In particular, the invention relates to an polypeptide

encoded by SEQ ID NO. 1 or encoded by a polynucleotide that has at least about 90 %, preferably at least about 95 %, most preferred about 98 % or more identity with SEQ ID NO. 1; preferably, such polypeptide has almost the same properties as human EDG 8; e.g. the same biological activity or functionality. One characteristic functionality of human EDG8 is that the polypeptide is a S1P receptor; it responds to S1P and optionally to related phospholipids like dHS1P or LPA as depicted and described in Fig. 2.

In an additional embodiment, a method of detecting a nucleic acid sequence encoding SEQ ID NO:1 in a biological sample comprising contacting a labeled nucleic acid probe that hybridizes with the nucleic acid sequence with the biological sample under conditions wherein the probe hybridizes with the nucleic acid sequence and detecting the hybridization of the probe to the nucleic acid sequence in the sample is provided.

By "biological sample" is meant any body fluid, tissue, cells or specimens obtained from a subject, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA from the biological samples may be used directly or may be amplified enzymatically by using PCR (Saiki et al., Nature 324:163-166, 1986) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding the G-protein coupled receptor protein can be used as a probe to identify and analyze G-protein coupled receptors. The probe is labeled according to methods well-known to the skilled artisan, which are described below. Similarly, the detecting is conducted under hybridization conditions that are known to the skilled artisan and are describe further, below.

In an additional embodiment, the invention relates to a kit comprising one or more containers, wherein at least one container contains a detectably labeled antibody that selectively binds a polypeptide encoded by SEQ ID NO:1. A kit comprising one or more containers, wherein at least one container contains a detectably labeled

nucleic acid probe that hybridizes under a stringency of 68°C with a polynucleotide encoding SEQ ID NO:2 is also provided.

The invention further relates to a polypeptide consisting of the amino acid sequence of SEQ ID NO. 2.

This invention is further related to a DNA sequence wherein the DNA sequence has been selected from at least one of the following group of polynucleotide sequences:
a) a polynucleotide comprising the polynucleotide sequence of SEQ ID No 1,
b) a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide sequence according to a),
c) a polynucleotide which hybridizes under low or medium stringency conditions to a polynucleotide sequence according to a), and
d) a polynucleotide sequence complementary to a polynucleotide sequence as defined in one of a), b), or c).

Thus, in one embodiment, the invention relates to an isolated nucleic acid molecule encoding a EDG8 polypeptide comprising SEQ ID NO:2. In another embodiment, the invention relates to an isolated nucleic acid molecule comprising SEQ ID NO:1, a nucleic acid molecule that is at least 95% or about 95% dentical to SEQ ID NO:1, a nucleic acid molecule that hybridizes under stringent conditions to one of the above or is complementary to one of the above. In another embodiment, the nucleic acid sequence consists of SEQ ID NO. 1.

The nucleic acid molecule of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule is intended a nucleic acid molecule, DNA or RNA, that has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include in vivo or in vitro RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules further includes such molecules produced synthetically.

In another embodiment, the DNA sequence as mentioned above can be part of the genome of each organism which harbors a gene for EDG8. In particular, the DNA sequence is part of a mammal or a human being.

It is understood that all nucleic acid molecules encoding EDG8 are also included herein, as long as they encode a polypeptide having the biological activity of human EDG8. By "EDG8 biological activity" is meant that the molecule is a functional receptor for S1P, LPA, dHS1P and related lysophospholipid mediators. Such activity may be assayed using well known techniques in the art. One such assay employs assessment of the ability of $Ca^{2+}$ to mobilize as described in Figure 2. Such nucleic acid molecules include naturally occurring, synthetic, and intentionally manipulated polynucleotides. For example, DNA encoding EDG8 may be subjected to site-directed mutagenesis. The nucleotide sequence for EDG8 also includes antisense sequences, and sequences encoding dominant negative forms of EDG8. The invention includes nucleotide sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of EDG8 polypeptide encoded by the nucleotide sequence is functionally unchanged. When the sequence is RNA, the deoxynucleotides A, G, C, and T of SEQ ID NO:1 are replaced by ribonucleotides A, G, C, and U, respectively.

The present invention also includes fragments of the above described nucleic acid molecule. For instance, fragments include a segment of contiguous nucleotides of SEQ ID NO:1, which are at least about 10 bases, preferably about 15 bases or about 20 bases or 30 bases, or 40 bases, or 50 bases in length. Such fragments are useful as diagnostic probes and PCR primers, as set forth herein. Of course, larger fragments of the nucleic acid molecules of the present invention also are contemplated. Fragments or portions of the polynucleotides of the present invention also may be used to synthesize full-length polynucleotides of the present invention.

For example, a nucleic acid probe may be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene of the present invention including regulatory and promoter regions, exons and introns. An example of a screen of this type comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the genes of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

As described above, fragments of the full length gene of the present invention may be used as hybridization probes for a cDNA or a genomic library to isolate the full length DNA and to isolate other DNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 10, preferably at least 15, and even more preferably at least 30 bases and may contain, for example, at least 50 or more bases. In fact, probes of this type having at least up to 150 bases or greater may be preferably utilized. The probe may also be used to identify a DNA clone corresponding to a full length transcript and a genomic clone or

clones that contain the complete gene including regulatory and promotor regions, exons and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary or identical to that of the gene or portion of the gene sequences of the present invention are used to screen a library of genomic DNA to determine which members of the library the probe hybridizes to.

It is also appreciated that such probes can be and are preferably labeled with an analytically detectable reagent to facilitate identification of the probe. Useful reagents include but are not limited to radioactivity, fluorescent dyes or enzymes capable of catalyzing the formation of a detectable product. The probes are thus useful to isolate complementary copies of DNA from other sources or to screen such sources for related sequences.

Thus, the present invention is directed to polynucleotides having at least about a about 70% identity, preferably at least about 90% identity and more preferably at least about a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2, as well as fragments thereof, which fragments have at least 15 bases, preferably at least 30 bases, more preferably at least 50 bases and most preferably fragments having up to at least 150 bases or greater, which fragments are at least about 90% identical, preferably at least about 95% identical and most preferably at least about 97% identical to any portion of a polynucleotide of the present invention.

In another embodiment, the invention relates to a nucleic acid molecule that hybridizes under stringent condition to SEQ ID NO:1. The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target complementary sequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and circumstance-dependent; for example, longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridization

with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993).

Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength pH. The Tm is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at TR, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

Exemplary, non-limiting stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1 % SDS, incubating at 42°C, or, 5x SSC, 1 SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. Alternative conditions include, for example, conditions at least as stringent as hybridization at 68°C. for 20 hours, followed by washing in 2X SSC, 0.1% SDS, twice for 30 minutes at 55°C. and three times for 15 minutes at 60°C. Another alternative set of conditions is hybridization in 6x at about 45°C, followed by one or more washes in 0.2x SSC, 0.1% SDS at 50-65°C. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C - 95°C for 30 sec - 2

min., an annealing phase lasting 30 sec. - 2 min., and an extension phase of about 72°C for 1 - 2 min.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

In an embodiment of the present invention, by "stringent" conditions is meant washing of filters in 0.1 X SSC, 0.1% SDS; 2 times for about 30 min. at about 68°C (or about 5°C below melting temperature). "Low medium" hybridization conditions means: washing of filters in 2 X SSC, 0.1% SDS; 2 times for about 30 min. at about 68°C (or about 10°C below melting temperature).

The polynucleotides which hybridize to the above described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of SEQ ID NO:1. For example, such polypeptide could function as a receptor for S1P and related compounds, viz., LPA, dHS1P and related lysophospholipid mediators. Such activity may be assayed using well known techniques in the art. One such assay employs assessment of ability of $Ca^{2+}$ mobilization in response to S1P mediated by the receptor, e.g., EDG8 or a functional fragment thereof, in CHO cell as described in Figure 2.

The nucleic acid molecule of the invention includes the DNA encoding SEQ ID NO:2 and conservative variations of SEQ ID NO:2. The term "conservative variation" as

used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

The nucleic acid molecule of the present invention can be derived from any mammal, particlarly humans. The preferred nucleic acid molecule is derived from humans. In the present invention, the nucleic acid molecule may be at least 95% or about 95% identical to SEQ ID NO:1. One of skill in the art can determine the percentage of sequence identity between two sequences by aligning the encoded amino acid sequences, determining the corresponding alignment of the encoding polynucleotides, and then counting the number of residues shared between the sequences being compared at each aligned position. No penalty is imposed for the presence of insertions or deletions, but insertion or deletions are permitted only where required to accommodate an obviously increased number of amino acid residues in one of the sequences being aligned. Offsetting insertions just to improve sequence alignment are not permitted at either the polypeptide or polynucleotide level. Thus, any insertions in the polynucleotide sequence will have a length which is a multiple of 3. The percentage is given in terms of residues in the test sequence that are identical to residues in the comparison reference sequence.

Percent identity is calculated for oligonucleotides of this length by not allowing gaps in either the oligonucleotide or the polypeptide for purposes of alignment. Whenever at least one of two sequences being compared is a degenerate oligonucleotide comprising an ambiguous residue, the two sequences are identical if at least one of the alternative forms of the degenerate oligonucleotide is identical to the sequence with which it is being compared. As an illustration, AYAAA is 100% identical to

ATAAA, since AYAAA is a mixture of ATAAA and ACAAA. Methods to determine the homology and percent identity of sequences are well known in the art. These methods can be performed manually (using mathematical calculations) or with a computer program, such as the Wisconsin package version 10.1-Unix (Genetics Computer Group (GCG), Madison, Wisconsin). Other methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.* 2: 482 (1981); by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970); by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci.* 8: 2444 (1988); by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California, GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 7 Science Dr., Madison, Wisconsin, USA; the CLUSTAL program is well described by Higgins and Sharp, *Gene* 73: 237-244 (1988); Higgins and Sharp, *CABIOS* : 11-13 (1989); Corpet, *et al., Nucleic Acids Research* 16: 881-90 (1988); Huang, *et al., Computer Applications in the Biosciences* 8: 1-6 (1992), and Pearson, *et al., Methods in Molecular Biology* 24: 7-331 (1994). The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, *Current Protocols in Molecular Biology*, Chapter 19, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995).

## ANTIBODIES

In another embodiment of the invention, the EDG8 polypeptides of the invention,

including fragments thereof, can be used to produce antibodies which are immunoreactive or bind to epitopes of the EDG8 polypeptides. Polyclonal antibodies and antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are encompassed by the invention.

The preparation of polyclonal antibodies is well-known to those skilled in the art. See, for example, Green et at., "Production of Polyclonal Antisera," in: Immunochemical Protocols pages 1-5, Manson, ed., Humana Press 1992; Coligan et al., "Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters," in: Current Protocols in Immunology, section 2.4.1, 1992, which are hereby incorporated by reference.

The preparation of monoclonal antibodies likewise is conventional. See, for example, Kohler & Milstein, Nature 256:495, 1975; Coligan et al., sections 2.5.1-2.6.7; and Harlow et al., in: Antibodies: a Laboratory Manual, page 726, Cold Spring Harbor Pub., 1988, which are hereby incorporated by reference. Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, e.g., Coligan et al., sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; Barnes et al., "Purification of Immunoglobulin G (IgG)," in: Methods in Molecular Biology, Vol. 10, pages 79-104, Humana Press, 1992.

Methods of in vitro and in vivo multiplication of monoclonal antibodies are well known to those skilled in the art. Multiplication in vitro may be carried out in suitable

culture media is such as Dulbecco's Modified Eagle Medium or RPMI 1640 medium, optionally supplemented by a mammalian serum such as fetal calf serum or trace elements and growth-sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, thymocytes or bone marrow macrophages. Production in vitro provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies. Large scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer reactor, or in immobilized or entrapped cell culture. Multiplication in vivo may be carried out by injecting cell clones into mammals histocompatible with the parent cells, e.g., syngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

Therapeutic applications for antibodies disclosed herein are also part of the present invention. For example, antibodies of the present invention may also be derived from subhuman primate antibody. General techniques for raising therapeutically useful antibodies in baboons can be found, for example, in Goldenberg et al., International Patent Publication WO 91/11465, 1991, and Losman et al., Int. J. Cancer 46:310, 1990, which are hereby incorporated by reference.

Alternatively, a therapeutically useful anti- EDG8 antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi et al., Proc. Natl. Acad. Sci. USA 86:3833, 1989, which is hereby incorporated in its entirety by reference. Techniques for producing humanized monoclonal antibodies are described, for

example, by Jones et al., Nature 321:522, 1986; Riechmann et al., Nature 332:323, 1988; Verhoeyen et al., Science 239:1534, 1988; Carter et al., Proc. Nat'l Acad. Sci. USA 89:4285, 1992; Sandhu, Crit. Rev. Biotech. 12:437, 1992; and Singer et al., J. Immunol. 150:2844, 1993, which are hereby incorporated by reference.

Antibodies of the invention also may be derived from human antibody fragments isolated from a combinatorial immunoglobulin library. See, for example, Barbas et al., in: Methods: a Companion to Methods in Enzymology, Vol. 2, page 119, 1991; Winter et al., Ann. Rev. Immunol. 12:433, 1994, which are hereby incorporated by reference. Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from STRATAGENE Cloning Systems (La Jolla, Calif.).

In addition, antibodies of the present invention may be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green et al., Nature Genet. 7:13, 1994; Lonberg et al., Nature 368:856, 1994; and Taylor et al., Int. Immunol. 6:579, 1994, which are hereby incorporated by reference.

The term "antibody" includes intact molecules as well as fragments thereof, such as Fab, $(Fab')_2$, and Fv which are capable of binding the epitopic determinant. These antibody fragments retain some ability to selectively bind with its antigen or receptor and are defined as follows:

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule can be produced by digestion of whole antibody with the enzyme

papain to yield an intact light chain and a portion of one heavy chain;

(2) Fab', the fragment of an antibody molecule can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3) $(Fab')_2$, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; $(Fab')_2$, is a dimer of two Fab' fragments held together by two disulfide bonds;

(4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5) Single chain antibody ("SCA"), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art. (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference). As used in this invention, the term "epitope" means any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

Antibodies which bind to the EDG8 polypeptide of the invention can be prepared using an intact polypeptide or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or a peptide used to immunize an animal can be derived from translated cDNA or chemical synthesis which can be conjugated to

a carrier protein, if desired. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), cmd tetanus toxoid. The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

If desired, polyclonal or monoclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (See for example, Coligan et al., Unit 9, Current Protocols in Immunology, Wiley Interscience, 1991, incorporated by reference).

It is also possible to use the anti-idiotype technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the "image" of the epitope bound by the first monoclonal antibody.

DIAGNOSTICS

Further, the invention relates to a process for diagnosing a disease or a susceptibility to a disease (such as cancer, angiogenesis and inflammation that implicates S1P in pathophysiological states of the diseases) (Pyne and Pyne, Biochem J 349(Part 2):385, 2000) related to expression or biological activity of EDG8 polypeptide comprising:

a) determining the presence or absence of mutation in the nucleotide sequence encoding said EDG8 polypeptide in the genome of said subject; and/or
b) analyzing for the presence or amount of the EDG8 polypeptide expression in a sample derived from said subject.

This invention is also related to the use of the nucleic acids encoding EDG8 as part of a diagnostic assay for detecting diseases or susceptibility to diseases related to the presence of mutated G-protein coupled receptor genes, such as EDG8 of SEQ ID NO:1. Such diseases are related to cell transformation, such as tumors and cancers.

Individuals carrying mutations in the human G-protein coupled receptor gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki et al., Nature 324:163, 1986) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding the EDG8 polypeptide can be used to identify and analyze EDG8 mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled EDG8 receptor RNA or alternatively, radiolabeled EDG8 receptor antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers et al., Science 230:1242, 1985).

Sequence changes at specific locations may also be revealed by nuclease protection

assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton et al., PNAS USA, 85:4397, 1985).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by in situ analysis.

The sequences of the present invention are also valuable for chromosome identification (see Table-1). The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments

from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include in situ hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

EXPRESSION VECTOR

The invention refers further to a vector, preferably a recombinant DNA expression vector. In one embodiment, the invention relates to a DNA vector comprising a nucleic acid molecule consisting of SEQ ID No. 1. The vector further comprises a polynucleotide element which renders the vector suitable for its multiplication in procaryotic or eucaryotic cells and a DNA sequence as aforementioned coding for the amino acid sequence or a polynucleotide sequence for EDG8. The term "expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the EDG8 genetic sequences. This DNA element which renders the vector suitable for multiplication can be an origin of replication which works in procaryotic or eucaryotic cells. An example for an origin of replication which works in procaryotic cells is the colE1 ori. A recombinant vector needs further a selection marker for control of growth of these organisms which harbor the vector. Suitable selection markers include genes which protect organisms from antibiotics (antibioticum resistance) e.g. ampicillin, streptomycin, chloramphenicol or provide growth under compound deprived environmental conditions (auxotrophic growth conditions) when expressed as proteins in cells. In a preferred embodiment of the invention for multiplication of the said recombinant vector the procaryotic cells are bacteria. In special preferred versions of the inventions the bacteria are in particular bacteria of Escherichia coli or of Bacillus spec.. In a further preferred embodiment of the invention for the multiplication of the

said recombinant vector the eucaryotic cells are cells of a cell line or yeast cells. In special preferred versions of the invention the cells of the cell line are cells of a CHO cell line.

Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

Thus, the nucleic acid sequence which encodes EDG8 can be operatively linked to expression control sequences. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (i.e., ATG) in front of a protein-encoding gene, splicing signal for introns, maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter. By "promoter" is meant minimal sequence sufficient to direct transcription. Thus, the said recombinant DNA of the present invention could provide for a promotor element which is operationally linked to a DNA sequence coding for the amino acid sequence or polynucleotide sequence of a EDG8 allowing transcription of the related RNA and/or expression of the related

protein. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the gene.These promotor elements can be taken in preferred versions of the invention from procaryotic promoters or eucaryotic promoters. A procaryotic promoter is characterized by its ability to induce transcription in procaryotic organisms as a eucaryotic promoter is characterized by its ability to induce transcription in eucaryotic organisms. Both procaryotic and eucaryotic promoter elements can be preferred inducible promoters or further preferred constitutive promoters (see e.g., Bitter et al., Methods in Enzymology 153:516-544, 1987). An inducible promoter is switched on only when a signal event is present. The signal can be born by the organism's metabolism. Then it often consists of metabolic products, hormones, degradation products of macromolecules or other metabolic derived substances. The signal can also be provided by the environment. Then it may consist of radiation, temperature or chemical compounds of the environment. A constitutive promoter needs no induction for activity. When cloning in bacterial systems, inducible promoters such as pL of bacteriophage, gamma, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

The invention includes further a host cell and a cell culture comprised of said host cells. This host cell comprising at least one recombinant DNA vector as mentioned before. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The cell may be prokaryotic or eukaryotic. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of

stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art. When the host cell is taken from procaryotic cells it preferably consists of a cell of a bacterium in particular of Escherichia coli or Bacillus spec. When this host cell consists of a eucaryotic cell it is preferred a cell of a cell line in particular a cell of a CHO cell line.

This host cell can be produced by transforming the said host cell by a recombinant DNA vector comprising a DNA sequence coding for an amino acid sequence or polynucleotide sequence of a EDG8. The transformation can take place by routine methods used in microbiology as for example transformation of competent cells, $Ca^{2+}$-phosphate-precipitation or electroporation. By "transformation" is meant a genetic change induced in a cell following incorporation of new DNA (i.e., DNA exogenous to the cell). Where the cell is a mammalian cell, the genetic change is generally achieved by introduction of the DNA into the genome of the cell (i.e., stable).

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding polypeptide of SEQ ID NO:1 or a fragment thereof. Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method using procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell if desired.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences of EDG8 of the invention. Another method is to use a eukaryotic viral

vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein (see for example, Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well know to those skilled in the art.

Isolation and purification of microbial expressed polypeptide, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

The EDG8 expressed polypeptides can be recovered and purified from recombinant host cells and cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

In one embodiment, the invention provides substantially purified polypeptide of SEQ ID No:2 or a fragment thereof. Preferably, EDG8 translated polypeptide has an amino acid sequence set forth in SEQ ID NO:2. The term "substantially purified" as

used herein refers to a polypeptide which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify EDG8-polypeptide using standard techniques for protein purification. The substantially pure polypeptide will yield a single major band on a non-reducing polyacrylamide gel. The purity of the polypeptide can also be determined by amino-terminal amino acid sequence analysis.

As explained above, the invention refers also to a protein encoded by one of the DNA sequences as aforementioned. This protein has activity of a EDG8. Activity of EDG8 is meant the molecule is a functional receptor for S1P, LPA, dHS1P and related lysophospholipid mediators. Such activity may be assayed using well known techniques in the art. One such assay employs assessment of ability of $Ca^{2+}$ mobilization as described in Figure 2. As described above, further included is production of a protein wherein first a host cell harboring a recombinant vector including a DNA sequence encoding for an amino acid sequence or a polynucleotide sequence for EDG8 is propagated in a suitable growth medium chosen from either media for bacteria or eucaryotic cells depending on the related host cell type. These propagated cells are second harvested by common methods of biochemistry as centrifugation or filtration and processed to obtain crude cell extracts. These cell extracts third are purified subsequently by methods used for protein purification as size exchange chromatography, ion exchange chromatography, affinity chromatography and others to gain the protein of interest (EDG8 activity) separated from other compounds of the cell lysates.

The polypeptide of the invention may be expressed in a modified form, such as a fusion protein and may include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent storage and handling. Also, peptide moieties may be added to the polypeptide to improve purification. Such regions may be removed prior to final preparation of the peptide. Thus, in one embodiment, the invention relates to a

fusion protein comprising a polypeptide consisting of the amino acid sequence of SEQ ID NO. 2. Additionally, the fusion protein of the invention could include amino acids of other members of the EDG family.

In one embodiment, the polypeptide of the present invention comprises the amino acid sequence of SEQ ID NO:2 and is encoded by the nucleotide sequence of SEQ ID NO:1. However, the polypeptide of the invention can be varied without significant effect on the structure or function of the molecule.

Minor modifications of the EDG8 primary nucleotide sequences may result in proteins which have substantially equivalent activity as compared to the unmodified counterpart polypeptide described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as the biological activity of the EDG8 still exists.

The polypeptide of the present invention also includes fragments and variants of SEQ ID NO:2. "Variant" when referring to the polypeptide of SEQ ID NO:2, means polypeptides which retain essentially the same biological function or activity as a polypeptide comprising the full length SEQ ID NO:2.

A "fragment" is a segment of SEQ ID NO:2 that comprises contiguous amino acids.

The variant of the polypeptide SEQ ID NO:2 may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptides are fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptides. Such variants are deemed to be within the scope of those skilled in

the art from the teachings herein.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 as well as polypeptides which have at least about 70% similarity to the polypeptide of SEQ ID NO:2 and more preferably about at least a 90% similarity to the polypeptide of SEQ ID NO:2 and still more preferably at least about a 95% similarity to the polypeptide of SEQ ID NO:2 and also includes fragments of such polypeptides with such portion of the polypeptide generally containing about at least 8 consecutive amino acids and preferably about at least 30 to 50 consecutive amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide. This can be done manually (using mathematical calculations) or with a computer program, such as the Wisconsin package version 10.1-Unix (Genetics Computer Group (GCG), Madison, Wisconsin).

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis, therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments also may be used to generate antibodies, as described above.

In addition, the invention relates to a method for identifying compounds which bind to EDG8 polypeptide comprising:

a) contacting a cell comprising the expression system or a part of such a cell with a candidate compound; and
b) assessing the ability of said candidate compound to bind to said cells.

Preferably, the method for identifying compounds further includes determining whether the candidate compound effects a signal generated by activation of the

EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of said signal is identified as an agonist.

In another embodiment of the invention, the method for identifying compounds further includes determining whether the candidate compound effects a signal generated by activation of the EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of said signal is identified as an antagonist.

SCREENING AND USES AS THERAPEUTICS

The present invention also relates to a method for determining whether a ligand not known to be capable of binding to a G-protein coupled receptor can bind to such receptor which comprises contacting a mammalian cell which expresses a G-protein coupled receptor with the ligand under conditions permitting binding of ligands to the G-protein coupled receptor, detecting the presence of a ligand which binds to the receptor and thereby determining whether the ligand binds to the G-protein coupled receptor. The systems hereinabove described for determining agonists and/or antagonists may also be employed for determining ligands which bind to the receptor. In the preferred embodiment , the receptor is EDG8.

In general, antagonists for G-protein coupled receptors which are determined by screening procedures may be employed for a variety of therapeutic purposes. For example, such antagonists have been employed for treatment of hypertension, angina pectoris, myocardial infarction, ulcers, asthma, allergies, psychoses, depression, migraine, vomiting, stroke, eating disorders, migraine headaches, cancer and benign prostatic hypertrophy.

Agonists for G-protein coupled receptors are also useful for therapeutic purposes, such as the treatment of asthma, Parkinson's disease, acute heart failure, hypotension, urinary retention, and osteoporosis.

Examples of G-protein coupled receptor antagonists include an antibody, or in some cases an oligonucleotide, which binds to the G-protein coupled receptor but does not elicit a second messenger response such that the activity of the G-protein coupled receptor is prevented. Antibodies include anti-idiotypic antibodies which recognize unique determinants generally associated with the antigen-binding site of an antibody. Potential antagonists also include proteins which are closely related to the ligand of the G-protein coupled receptor, i.e. a fragment of the ligand, which have lost biological function and when binding to the G-protein coupled receptor, elicit no response.

The invention also relates to an agonist or antagonist identified by such methods.

In another special embodiment of the invention, the method further includes contacting said cell with a known agonist for said EDG8 polypeptide; and determining whether the signal generated by said agonist is diminished in the presence of said candidate compound, wherein a candidate compound which effects a diminution in said signal is identified as an antagonist for said EDG8 polypeptide. The known agonist is for example S1P, LPA and/or dHS1P. The invention also relates to an antagonist identified by the method.

A compound can affect EDG8 by either stimulating or inhibiting EDG8 activity. An antagonist is a compound that directly or indirectly "inhibits" a signal generated by activation of the EDG8 polypeptide at the surface of the cell. An agonist is a compound that directly or indirectly "stimulates" a signal generated by activation of the EDG8 polypeptide.

Potential antagonists to the EDG8 polypeptides of the present invention include an antibody against the EDG8 polypeptides, or in some cases, an oligonucleotide, which bind to the EDG8 polypeptides and alter its conformation.

Potential antagonists also include antisense constructs produced by antisense

technology. Antisense technology controls gene expression through triple-helix formation, etc. The number of EDG8 may be reduced through antisense technology, which controls gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix--see Lee et al., Nucl. Acids Res., 6:3073, 1979); Cooney et al, Science, 241:456, 1988); and Dervan et al., Science, 251: 1360, 1991), thereby preventing transcription and the production of the EDG8 polypeptides. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into the EDG8 polypeptides (antisense--Okano, J. Neurochem., 56:560, 1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, Fla. 1988). The antisense constructs can be delivered to cells by procedures known in the art such that the antisense RNA or DNA may be expressed in vivo. -

The antagonist or agonist compounds may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the compound, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention in addition, relates to a method of preparing a pharmaceutical composition comprising:

a) identifying a compound which is an agonist or an antagonist of EDG8,

b) preparing the compound, and

c) optionally mixing the compound with suitable additives.

The invention also relates to a pharmaceutical compound prepared by such a process.

The invention relates to a pharmaceutical, comprising as active ingredient for example such identified compound, an EDG8 polypeptide or a polynucleotide encoding for EDG8 or a part thereof.

In particular, the invention relates to a pharmaceutical, that can be used for the prevention and/or treatment of diseases associated with EDG8/S1P signal transduction, for example diseases associated with endothelial dysfunction such as for example Atheriosclerosis, Shoke, Hypertonie, coronary syndroms, cancer, thrombolylic diseases, affected wound healing and diseases accompanied by increased cell death. In another aspect of the invention, such pharmaceutical can be used for the prevention and/or treatment of diseases associated with a dysregulation of angiogenesis, such as for example tumor growth, rheumatical arthritis and diabetic setinopathy.

The study, reported about the cloning, chromosomal mapping, tissue expression and functional identification as a receptor for S1P of a novel GPCR, EDG8, the fifth functional receptor for sphingosine 1-phosphate.

In an effort to identify new G-protein coupled receptors of the EDG-family a database search with alignments of the currently known 18 members of this receptor family was performed, comprising human EDG1-7 sequences up to the putative EDGs from Xenopus and Zebra-fish. A multiple alignment of these sequences was created by CLUSTALW and used in a PSI-BLAST search to scan translated versions of human genomic DNA sequences, which were publicly available in the different EMBL sections. For translation of DNA into protein sequences, individual protein files within two respective STOP-codon were created and all proteins shorter than 50 amino acids were ignored. As the majority of GPCRs is unspliced searching for GPCRs within genomic sequences should bring about novel receptor proteins.

Performing a PSI-BLAST search, the various cDNAs and genomic contigs, respectively, for the human EDG1-7 receptors were identified, and an additional genomic hit, highly homologous to human EDG5 (51% homology), termed EDG8. The nucleotide and amino acid sequence of the new putative GPCR are depicted in Fig.1A. Hydropathy analysis (hydrophobicity plot not shown) suggests a seven transmembrane protein with three alternating extra- and intracellular loops, assumed to be the heptahelix structure common to GPCRs.

To shed more light on the relationships involved in the molecular evolution of the EDG-receptor family, a grow tree phylogram was constructed using the neighbor joining method (Genetic Computer Group (GCG), Madison, Wisconsin. (Fig.1B) Comparison of amino acid sequences). According to this phylogenetic tree, the human EDG-family can be divided into two distinct groups: EDG1, 3, 5 and 6 belonging to one, EDG2, 4 and 7 belonging to the other group. These two groups are discriminated further by their preference for different lipid ligands: EDG1, 3, 5, 6 are preferentially stimulated by sphingosin 1-phosphate (S1P) (Yatomi et al., J Biochem (Tokyo) 12:969, 1997; Lee et al., Science 279:1552, 1998; Lee et al., J Biol Chem 273:22105, 1998; Ancellin and Hla, J Biol Chem 274:18997, 1999; Yamazaki et al., Biochem Biophys Res Commun 268:583, 2000; Van Brocklyn et al., Blood 95:2624, 2000), EDG2, 4 and 7 by lysophosphatidic acid (LPA) (Hecht et al., 1996; An et al., J Biol Chem 273:7906, 1998; Im et al., Mol Pharmacol 57:753, 2000). The newly identified EDG8 exhibited highest similarity (86.8% amino acid identity) to the rat nrg1-protein (Fig. 1B), a GPCR recently cloned by EST-expression profiling from a rat PC12 cell library (Glickman et al., Mol Cell Neuroscience 14:141, 1999), which probably represents the rat homologue of human EDG8. In the report of Glickman et al., however, the authors did not address the question of the activating ligand of this receptor. The high similarity between EDG8 and the known sphingosin 1-phosphate (S1P) receptors EDG1, 3 and 5 (48-51%) (Fig. 1C) led to test the hypothesis that EDG8 may be a functional S1P-receptor.

In testing for S1P receptor activity, the EDG8 cDNA was introduced into chinese hamster ovary (CHO) cells by transient transfection. CHO cells were chosen as they exhibit minimal responses to sphingosin 1-phosphate in concentrations up to 1 μM but respond to S1P after transfection with the S1P preferring receptors EDG 1, 3 and 5 (Okamoto et al., J Biol Chem 273:27104, 1998; Kon et al., J Biol Chem 274:23940, 1999). To test functional receptor activity the mobilization of $[Ca^{2+}]_i$ was monitored for three reasons:

1) S1P has been reported to increase $Ca^{2+}$ in many cell types (Ghosh et al., 1990; Zang et al., 1991; Durieux et al., Am J Physiol 264:C1360, 1993; Chao et al., J Biol Chem 269:5849, 1994; Gosh et al., J Biol Chem 269:22628, 1994; Mattie et al., J Biol Chem 269:3181, 1994; Meyer zu Heringdorf et al., Naunyn-Schmiedeberg's Arch Pharmacol 354:397, 1997; Okajima et al., FEBS Lett 379:260, 1996; van Koppen et al., J Biol Chem 271:2082, 1996; Törnquist et al., Endocrinology 138:4049, 1997; Yatomi et al., J Biochem (Tokyo) 12:969, 1997; Noh et al., J Cell Physiol 176:412, 1998; An et al., Mol Pharmaco 55:787,1999).

2) the identification of EDG1, 3, 5 and 6 as receptors for S1P has provided the molecular basis for a GPCR mediated mechanism and the receptors are known to mediate intracellular $Ca^{2+}$-release through either PTX-sensitive $G\alpha_i$ proteins or the PTX-insensitive $G\alpha_{q/11}$ pathway (Okamoto et al., J Biol Chem 273:27104, 1998; Kon et al., J Biol Chem 274:23940, 1999; Gonda et al., Biochem J 337:67, 1999).

3) all currently known S1P-responding EDG-receptors (except EDG6) are present in endothelial cells (A. Niedernberg et al., submitted), in which intracellular $Ca^{2+}$ release is a major pathway in the generation of NO, an important factor in vascular biology. Thus, identification of the complete set of S1P receptors, involved in intracellular $Ca^{2+}$ mobilization could help clarify the role of the individual subtypes in endothelial cell signaling.

Fig.2 depicts measurement of the intracellular $Ca^{2+}$ concentration, mediated by S1P via the putative S1P receptor EDG8. For sake of comparison, the S1P-receptors EDG1, 3, 5, and 6, which have been reported to mobilize $[Ca^{2+}]_i$, were included. $[Ca^{2+}]_i$ were recorded as real time measurements using the Fluorescence plate imaging reader (FLIPR, Molecular Devices). Initially, CHO cells transfected with empty vector DNA were stimulated with different concentrations of S1P (10, 100, 1000 nM). None of the applied S1P concentrations was capable of eliciting significant rises in intracellular $Ca^{2+}$ (Fig. 2A), suggesting that S1P receptors are not expressed in CHO cells or, if expressed, are unable to signal via the endogeneous $G\alpha_q$ pathway. To address this issue, the G protein chimera $G\alpha_{qi5}$, which confers onto Gi coupled receptors the ability to stimulate the Gq pathway, and $G\alpha_{16}$, which links Gi- and Gs coupled receptors to PLCß and subsequent intracellular $Ca^{2+}$-mobilization were used. Upon stimulation with S1P, $G_{qi5}$- and $G_{16}$- transfected CHO cells did not give rise to significant increases in $[Ca^{2+}]_i$ (Fig. 2A). However, transient transfection of CHO-cells with the cDNAs coding for the EDG1, 3 and 5 receptor conferred S1P-responsiveness to the cells: it was confirmed that EDG1, 3 and 5 mobilize $[Ca^{2+}]_i$ in response to S1P (Fig. 2B, C, D) (Kon et al., J Biol Chem 274:23940, 1999). As already known for a large number of Gq-coupled receptors, coexpression of $G\alpha_q$ augments the EDG1 and 5-mediated $Ca^{2+}$-response as compared with the $Ca^{2+}$ signal induced by stimulation of endogeneous $G\alpha_q$. In case of EDG3, additional exogeneously added $G\alpha_q$ did not further improve the signal intensity. These results are in agreement with the findings reported by Kon et al. (J Biol Chem 274:23940, 1999), who showed that the EDG3-subtype causes the most robust enhancement of intracellular $Ca^{2+}$.

In case of EDG6, Yamazaki et al. (Biochem Biophys Res Commun 268:583, 2000) obtained an S1P-induced mobilization of $[Ca^{2+}]_i$ but in this study, investigators failed to detect a significant $Ca^{2+}$ increase above basal levels in the absence of any cotransfected G-protein α subunit (Fig. 2E). The reason for this discrepancy could

be the cellular background (CHO cells in this study vs. K562 cells in Yamazaki et al., Biochem Biophys Res Commun 268:583, 2000), as they reported a pertussis toxin (PTX)-sensitive $Ca^{2+}$-response, indicating the involvement of Gi-type G-proteins. In this case the $Ca^{2+}$ signal would be elicited by ßγ, released from activated $G\alpha_i ß\gamma$ heterotrimers. The $G\alpha_i$-induced $Ca^{2+}$ signals are known to be much smaller in intensity as compared with the $Ca^{2+}$ signals induced by bona-fide Gq-linked receptors (Kostenis et al., J Biol Chem 272:19107, 1997). It may be that detection of such $[Ca^{2+}]_i$ concentrations is beyond the sensitivity of the FLIPR system.

EDG8 did not release $[Ca^{2+}]_i$ when stimulated with S1P (10, 100, and 1000 nM) (Fig.2F), but gained the ability to mobilize $Ca^{2+}$ upon cotransfection with $G\alpha_{16}$, a G-protein α subunit, known to couple GPCRs from different functional classes to the Gq-PLCß pathway or $G\alpha_{qi5}$, a mutant G-protein α subunit that confers onto Gi-linked receptors the ability to stimulate Gq (Conklin et al., 1993). These results show that EDG8 is a functional receptor for S1P and that EDG8-induced $Ca^{2+}$ responses are due to a non-Gq pathway, probably the activation of phospholipase Cß2 by ßγ subunits of the Gi proteins. Furthermore, these results provide additional evidence that the S1P-preferring EDG-receptors couple differentially to the Gq and Gi pathways: EDG3 ist the most potent $Ca^{2+}$-mobilizing receptor and overexpression of $G\alpha_q$ does not further improve $Ca^{2+}$ signalling; EDG1 and 5 induce moderate $Ca^{2+}$-increases, that can be significantly improved by cotransfection of $G\alpha_q$ or a chimeric $G\alpha_{qi5}$ protein; EDG8-mediated $Ca^{2+}$-responses require cotransfection of $G\alpha_{qi5}$ or $G\alpha_{16}$.

To check whether the EDG8 receptor also reacts to related lysophospholipid mediators, the inventors examined the abilities of lysophosphatidic acid (LPA), dihydrosphingosin 1-phosphate (dHS1P), sphingosylphosphorylcholine (SPC) and lysophosphatidylcholine (LPC) to increase intracellular $Ca^{2+}$ in CHO cells transiently transfected with the EDG8 receptor and the G-protein α subunits $G\alpha_{16}$

and $G\alpha_{qi5}$ (Fig.3). Besides S1P, which was the most potent activator of EDG8, LPA and dHS1P evoked $[Ca^{2+}]_i$ increases in concentrations of 100 and 1000 nM. SPC and LPC, respectively, failed to generate any significant response in concentrations up to 1 µM. These data show that EDG8 is a S1P preferring receptor, but also responds to related phospholipids like dHS1P or LPA, as has also been reported for EDG1, which is a high affinity receptor for S1P and a low affinity receptor for LPA (Lee et al., J Biol Chem 273:22105, 1998). Therefore, EDG8 receptor has the characteristic functionality to respond to S1P and related phospholipids like DMS 1P or LPA. The response to S1P and other related phospholipides can for example be determined as described in Example 3. Cells containing the respective Gα can be obtained as described in Example 2.

Next, the expression pattern of the EDG8 gene in human tissues was investigated by Northern blot analysis (Fig.4). Tissues positive for EDG8 RNA were skeletal muscle, heart and kidney, lower abundance of RNA was seen in liver and placenta, no signal was detected in brain, thymus, spleen, lung and peripheral blood leukocytes. In all tissues a single RNA transcript of 5.5 kb was observed after hybridization with a DIG-labelled EDG8 antisense RNA probe. EDG8 exhibits highest similarity to the rat nrg1-GPCR (Glickman et al., Mol Cell Neuroscience 14:141, 1999) with an amino acid identity of 86.8% (Fig.1B) suggesting that it may be the human homolog of the rat nrg1 protein. However, the expression pattern of human EDG8 is quite different from the rat nrg1-receptor, which is found almost exclusively in brain (Glickman et al., Mol Cell Neuroscience 14:141, 1999). This finding suggests that EDG8 may represent a closely related but entirely different receptor from nrg1, rather than the human homolog. Never the less, it does not rule out the possibility that EDG8 and nrg1 are homologs with entirely different, species-dependent expression patterns.

As the first member of the EDG-family of GPCRs - EDG1 - was originally cloned as an endothelial differentiation gene from phorbol-myristic-acetate-treated differentiating human endothelial cells (Hla and Maciag, J Biol Chem 265:9308, 1990) and subsequently cloned from a human umbilical vein endothelial cell library

exposed to fluid shear stress as an upregulated gene it is reasonable to assume that EDG receptors play an important role in the regulation of endothelial function. Therefore, the presence of EDG8 transcripts in several human endothelial cell lines was analyzed. RT-PCR analysis of human umbilical vein endothelial cells (HUVECs), human coronary artery endothelial cells (HCAECs), human microvascular endothelial cells of the lung (HMVEC-L) and human pulmonary artery endothelial cells (HPAEC) revealed EDG8 expression in all cell lines tested (Fig.5A). In Fig.5B it is shown that EDG8 specific primers indeed solely amplify EDG8 sequences and none of the related EDG1-7 sequences. These findings suggest that the presence of EDG8 in different peripheral organs may be due to its localization in endothelial cells; it does not rule out, however, that EDG8 transcripts occur in cell types other than endothelial cells.

The expression of EDG8 in addition to EDG1, 3, and 5 (Rizza et al., Laboratory Investigation 79:1227, 1999) in HUVECs and several other endothelial cell lines is intriguing in view of all the reports regarding S1P effects on endothelial cell signalling. Hisano et al. (Blood 93:4293, 1999) reported that S1P protects HUVECs from apoptosis induced by withdrawal of growth factors and stimulates HUVEC DNA synthesis; the authors derived a model for cell-cell interactions between endothelial cells and platelets but the S1P-receptor responsible for HUVEC-protection of apoptosis could not be identified. Rizza et al. (Laboratory Investigation 79:1227, 1999) reported that S1P plays a role in endothelial cell leukocyte interaction in that S1P induces expression of cell adhesion molecules in human aortic endothelial cells, allowing monocytes and neutrophils to attach. These effects were blocked by pertussis toxin, suggesting the involvement of a Gi-coupled S1P receptor. The responsible S1P-receptor subtype, however, could not be identified and the EDG8 receptor was not included at the time of this study. Expression profiling of all EDG receptors in individual cell lines and the use of EDG receptor subtype selective compounds will clearly be necessary to help determine the role of the individual S1P receptors in endothelial cell signalling mechanisms.

Finally, the mapping of EDG receptors in genomic sequences allowed to derive the chromosomal localization for four genes of this family (Table 1). Interestingly, so far, four EDG-receptors including EDG8 are located on chromosome 19. In addition, the genomic sequence allowed the determination of the structure of the genes: the S1P-preferring receptors EDG1, 3, 5 and 8 are intronless as opposed to the LPA-preferring subtypes 2, 4 and 7, that contain an intron in the open reading frame in TMVI. These data suggest that in addition to the activating ligand and the degree of homology, the two subclasses of lysophospholipid receptors can be discriminated further by their genomic structure. The genomic structure of new potential EDG/LPA-receptor family members may also help predict the nature of the activating lipid ligand.

In conclusion, a new member of the EDG-family of G-protein coupled receptor, human EDG8, was isolated. This receptor functions as a cellular receptor for sphingosine 1-phosphate. EDG8 could exclusively be detected in peripheral tissues like skeletal muscle, heart and kidney and several human endothelial cell lines. It is conceivable that the expression in endothelial cells may account for the broad tissue distribution of this receptor. The existence of at least eight EDG-receptors for lysophospholipids suggests that receptor subtype selective agonists and antagonists will essentially be necessary for a better understanding of the biology of lysophospholipids and their respective receptors.

TABLE 1: Chromosomal localization, gene structure and accession number of the respective EDG genomic clones. Mapping of EDG receptors in genomic sequences allowed to derive a chromosomal assignment for EDG1, 2, 4-8. The chromosomal localization of EDG3 was obtained from Yamaguchi et al. (1996). Genomic sequences also revealed EDG1, 3, 5, 6 and 8 to be unspliced as opposed to EDG2, 4 and 7, which contain an intron in their open reading frame (ORF).

| EDG | Chromosomal localization | spliced/unspliced in ORF | according BAC accession number: |
|---|---|---|---|
| EDG1 | 1p21.1-21.3 | unspliced | AL161741 |
| EDG2 | 9q31.1-32/ /18p11.3 | spliced | AL157881/ /AP000882 |
| EDG3 | 9q22.1-q22.2 | unspliced | |
| EDG4 | 19p12 | spliced | NT_000939 |
| EDG5 | 19 | unspliced | AC011511 |
| EDG6 | 19p13.3 | unspliced | AC011547 |
| EDG7 | 1p22.3-31.2 | spliced | AL139822 |
| EDG8 | 19 | unspliced | AC011461 |

EXAMPLE 1: Molecular cloning of the human EDG8 receptor

As the putative human EDG8 sequence is intronless, the receptor was cloned from human genomic DNA (CLONTECH, Palo Alto, CA, 94303-4230) via polymerase chain reaction (PCR). PCR conditions, established to amplify the EDG8 sequence were 94°C, 1 min followed by 35 cycles of 94°C, 30sec, 68°C, 3 min, using GC-Melt Kit (CLONTECH, Palo Alto,CA). Primers designed to amplify the EDG8 sequence contained a HindIII site in the forward, and a EcoRI site in the reverse primer, respectively. The 1197 bp PCR product was cloned into the pCDNA3.1(+) mammalian expression vector (Invitrogen, Carlsbad, California) and sequenced in both directions.

EXAMPLE 2:Cell culture and Transfection

CHO-K1 cells were grown in basal ISCOVE medium supplemented with 10 % fetal bovine serum at 37°C in a humidified 5 % $CO_2$ incubator. For transfections, 2 x $10^5$ cells were seeded into 35-mm dishes. About 24 hr later cells were transiently transfected at 50-80 % confluency with the indicated receptor and G-protein constructs (1µg of plasmid DNA each) using the Lipofectamine transfection reagent and the supplied protocol (GIBCO). 18-24 hr after transfection cells were seeded into 96well plates at a density of 50.000 cells per well and cultured for 18-24 additional hr until used in the functional FLIPR assays.

The cDNA for Gα16 was cloned from TF1 cells by RT-PCR and ligated into the pCDNA1.1 mammalian expression vector (Invitrogen). Murine wild type Gαq was cloned from cells by RT-PCR and inserted into the BamHI-NsiI-sites of pCDNA1.1. To create the C-terminally modified $G\alpha_{qi5}$ subunit, in which the last five aa of wt Gαq were replaced with the correspoding $G\alpha_i$ sequence, a 175-bp BglII-NsiI fragment was replaced, in a two piece ligation, with a synthetic DNA fragment, containing the desired codon changes. The correctness of all PCR-derived sequences was verified by sequencing in both directions.

EXAMPLE 3: Fluorometric Imaging Plate Reader (FLIPR) Assay

Twenty-four hours after transfection, cells were splitted into 96-well, black-wall microplates (Corning) at a density of 50,000 cells per well. 18-24 hr later, cells were loaded with 95µl of HBSS containing 20 mM Hepes, 2.5 mM probenecid, 4 µM fluorescent calcium indicator dye Fluo4 (Molecular Probes) and 1 % fetal bovine serum for 1 h(37°C, 5 % $CO_2$). Cells were washed three times with HBSS containing 20 mM Hepes and 2.5 mM probenecid in a cell washer. After the final wash, the solution was aspirated to a residual volume of 100 µl per 96 well. Lipid ligands were dissolved in DMSO as 2 mM stock solutions (treated with ultrasound when necessary) and diluted at least 1:100 into HBSS containing 20 mM HEPES, 2.5 mM probenecid and 0.4 mg/ml fatty acid free bovine serum albumine. Lipids were aliquoted as 2X solutions into a 96 well plate prior to the assay. The fluorometric imaging plate reader (FLIPR, Molecular Devices) was programmed to transfer 100 µl from each well of the ligand microplate to each well of the cellplate and to record fluorescence during 3 min in 1 second intervals during the first minute and 3 second intervals during the last two minutes. Total fluorescence counts from the 18-s to 37-s time points are used to determine agonist activity. The instrument software normalizes the fluorescent reading to give equivalent initial readings at time zero.

EXAMPLE 4: Northern Blot analysis

Human multiple tissue Northern blots were purchased from CLONTECH (Palo Alto, CA, 94303-4230, USA). Antisense RNA probes were generated by subcloning nucleotides 279-1197 of the coding region into the Bam HI-Eco RI sites of the expression vector PSPT18 (Roche Diagnostics, Mannheim, Germany) and subsequent random priming with a DIG-RNA Labeling kit (Roche Diagnostics, Mannheim, Germany), using T7 RNA polymerase. Hybridization was carried out at 68°C for 16 h in hybridization buffer (Dig Easy Hyb Roche Diagnostics, Mannheim, Germany). Each blot was washed , blocked and detected as indicated in the standard protocol with the DIG Wash and Block Buffer set (Roche Diagnostics,

Mannheim, Germany) and treated with 1 ml CSPD ready–to-use(Roche Diagnostics, Mannheim, Germany) for 15 min , 37°C and developed for 5 min on the Lumiimager (Roche). Finally, each blot was stripped (50 % formamide, 5 % SDS, 50 mM Tris/HCl pH 7,5 ; 80° C, 2x 1 hour) and rehybridized with a GAPDH anti-sense RNA probe as an internal standard.

EXAMPLE 5: RNA Extraction and RT-PCR

RNA was prepared from different endothelial cell lines (HUVECS, HCAEC, HMVEC-L, HPAEC) using the TRIzol reagent (Hersteller, Lok.). Briefly, for each endothelial cell line, cells of a subconfluent 25 cm2 tissue culture flask were collected in 2,5ml TRIzol and total RNAs were extracted according to the supplied protocol. The purity of the RNA preparation was checked by veryfying the absence of genomic DNA. An aliquot of RNA, corresponding to ~5µg, was used for the cDNA generation using MMLV reverse transcriptase and the RT-PCR kit from STRATAGENE. RT-PCR was carried out in a volume of 50 µl, the RT-PCR conditions were set to 65°C for 5 min, 15min at RT, 1 hour at 37°C, 5 min at 90°C, chill on ice.
The cDNA templates for the PCR reactions (35 cycles of 94°C for 30 sec, 68°C for 3 min) were the reverse transcribed products of RNAs isolated from human endothelial cell lines (HUVECS,HCAEC, HMVEC-L, HPAEC). Typically, 1-5 µl of reverse transcribed cDNAs were used as templates for the PCR reactions.

EXAMPLE 6:Sources of materials

1-oleoyl-LPA, sphingosin 1-phosphate (S1P), dihydrosphingosin 1-phosphate (dHS1P), lysophosphatidylcholine (LPC), sphingosylphosphorylcholine (SPC) and fatty acid free BSA were from SIGMA (P.O.Box 14508, St. Louis, Missouri 63178). CHO-K1 cells were obtained from the American Type culture collection (ATCC, Manassas, Virginia), cell culture media and sera from GIBCO BRL (Gaithersburg, MD), the Ca fluorescent dye FLUO4 and pluronic acid from Molecular devices (Sunnyvale CA 94089-1136,USA) human northern blot membrane from CLONTECH (1020 East Meadow Circle, Palo Alto, California 94303-4230, USA.), commercially

available cDNAs (heart, fetal heart, left atrium, left ventricle, kidney, brain, liver, lung, aorta) from Invitrogen, oligonucleotides from MWG-Biotech AG (Ebersberg, Germany), the RT-PCR kit from SIGMA, the GC-melt PCR kit from Clontech (Palo Alto, CA), the expression plasmid pcDNA3.1 for EDG8 and pCDNA1.1 for expression of G-protein $\alpha$ subunits from Invitrogen (Carlsbad, CA 92008), competent DH5$\alpha$ from GIBCO and MC 1063 from Invitrogen.

All citations, including patents, patent applications, journal articles, books and other publications are herein incorporated by reference in their entirety.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 2

(2) INFORMATION FOR SEQUENCE ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) TYPE: Nucleic acid. Nucleotide sequence of human EDG8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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  61 TACACCGGCAAGCTCCGCGGTGCGCGCTACCAGCCGGGTGCCGGCCTGCGCGCCGACGCC
 121 GTGGTGTGCCTGGCGGTGTGCGCCTTCATCGTGCTAGAGAATCTAGCCGTGTTGTTGGTG
 181 CTCGGACGCCACCCGCGCTTCCACGCTCCCATGTTCCTGCTCCTGGGCAGCCTCACGTTG
 241 TCGGATCTGCTGGCAGGCGCCGCCTACGCCGCCAACATCCTACTGTCGGGGCCGCTCACG
 301 CTGAAACTGTCCCCCGCGCTCTGGTTCGCACGGGAGGGAGGCGTCTTCGTGGCACTCACT
 361 GCGTCCGTGCTGAGCCTCCTGGCCATCGCGCTGGAGCGCAGCCTCACCATGGCGCGCAGG
 421 GGGCCCGCGCCCGTCTCCAGTCGGGGGCGCACGCTGGCGATGGCAGCCGCGGCCTGGGGC
 481 GTGTCGCTGCTCCTCGGGCTCCTGCCAGCGCTGGGCTGGAATTGCCTGGGTCGCCTGGAC
 541 GCTTGCTCCACTGTCTTGCCGCTCTACGCCAAGGCCTACGTGCTCTTCTGCGTGCTCGCC
 601 TTCGTGGGCATCCTGGCCGCTATCTGTGCACTCTACGCGCGCATCTACTGCCAGGTACGC
 661 GCCAACGCGCGGCGCCTGCCGGCACGGCCCGGGACTGCGGGGACCACCTCGACCCGGGCG
 721 CGTCGCAAGCCGCGCTCGCTGGCCTTGCTGCGCACGCTCAGCGTGGTGCTCCTGGCCTTT
 781 GTGGCATGTTGGGGCCCCCTCTTCCTGCTGCTGTTGCTCGACGTGGCGTGCCCGGCGCGC
 841 ACCTGTCCTGTACTCCTGCAGGCCGATCCCTTCCTGGGACTGGCCATGGCCAACTCACTT
 901 CTGAACCCCATCATCTACACGCTCACCAACCGCGACCTGCGCCACGCGCTCCTGCGCCTG
 961 GTCTGCTGCGGACGCCACTCCTGCGGCAGAGACCCGAGTGGCTCCCAGCAGTCGGCGAGC
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1021 GCGGCTGAGGCTTCCGGGGGCCTGCGCCGCTGCCTGCCCCCGGGCCTTGATGGGAGCTTC
1081 AGCGGCTCGGAGCGCTCATCGCCCCAGCGCGACGGGCTGGACACCAGCGGCTCCACAGGC
1141 AGCCCCGGTGCACCCACAGCCGCCCGGACTCTGGTATCAGAACCGGCTGCAGACTGA
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(2) INFORMATION FOR SEQUENCE ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) TYPE: Amino acid sequence of human EDG8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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M E S G L L R P A P V S E V I V L H Y N
Y T G K L R G A R Y Q P G A G L R A D A
V V C L A V C A F I V L E N L A V L L V
L G R H P R F H A P M F L L L G S L T L
S D L L A G A A Y A A N I L L S G P L T
L K L S P A L W F A R E G G V F V A L T
A S V L S L L A I A L E R S L T M A R R
G P A P V S S R G R T L A M A A A A W G
V S L L L G L L P A L G W N C L G R L D
A C S T V L P L Y A K A Y V L F C V L A
F V G I L A A I C A L Y A R I Y C Q V R
A N A R R L P A R P G T A G T T S T R A
R R K P R S L A L L R T L S V V L L A F
V A C W G P L F L L L L L D V A C P A R
T C P V L L Q A D P F L G L A M A N S L
L N P I I Y T L T N R D L R H A L L R L
V C C G R H S C G R D P S G S Q Q S A S
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A A E A S G G L R R C L P P G L D G S F

S G S E R S S P Q R D G L D T S G S T G

S P G A P T A A R T L V S E P A A D *